RICE AND PRODUCTS THEREOF HAVING STARCH WITH AN INCREASED PROPORTION OF AMYLOSE

FIELD OF THE INVENTION

5 This invention relates to a rice plant having kernel starch with a high relative amylose content. The invention also relates to rice with a reduced starch branching enzyme IIa (SBEIIa) activity in the endosperm. The invention also relates to grain and starch and food and non-food products obtained therefrom.

10 ## BACKGROUND OF THE INVENTION
Cereal starch comprises two types of molecule, amylose and amylopectin. Amylose is an essentially linear molecule composed of α-1,4 linked glucosidic units, while amylopectin is highly branched with α-1,6 glucosidic bonds linking linear chains.

15 The synthesis of starch in the endosperm of higher plants is carried out by a suite of enzymes that catalyse four key steps. Firstly, ADP-glucose pyrophosphorylase (ADGP) activates the monomer precursor of starch through the synthesis of ADP-glucose from G-1-P and ATP. Secondly, the activated glucosyl donor, ADP-glucose, is transferred to the non-reducing end of pre-existing α–1,4 linked chains by starch synthases. Thirdly, starch
20 branching enzymes (SBE) introduce branch points through the cleavage of a region of α-1,4 linked glucan followed by transfer of the cleaved chain to an acceptor chain, forming a new α-1,6 linkage. SBEs are the only enzymes that can introduce the α-1,6 linkages into α-polyglucans and therefore play an essential role in the formation of amylopectin. Finally, starch debranching enzymes remove some of the branch linkages, although the
25 mechanism through which they act is unresolved (Myers *et al.*, 2000). While it is clear that at least these four activities are required for normal starch granule synthesis in higher plants, multiple isoforms of each of the four activities are found in the endosperm of higher plants and specific roles have been proposed for individual isoforms on the basis of mutational analysis (Wang *et al*, 1998a, Buleon *et al.*, 1998) or through the modification
30 of gene expression levels using transgenic approaches (Abel *et al.*, 1996, Jobling *et al.*, 1999, Schwall *et al.*, 2000). However, the precise contributions of each isoform of each activity to starch biosynthesis are still not known, and it is not known whether these contributions differ markedly between species.

35 In the cereal endosperm, two isoforms of ADP-glucose pyrophosphorylase are present, one form within the amyloplast, and one form in the cytoplasm (Denyer *et al.*, 1996,

Thorbjornsen *et al.*, 1996). Each form is composed of two subunit types. The shrunken (*sh2*) and brittle (*bt2*) mutants in maize represent lesions in large and small subunits respectively (Giroux and Hannah, 1994). Four classes of starch synthase are found in the cereal endosperm, an isoform exclusively localised within the starch granule, granule-

5   bound starch synthase (GBSS), two forms that are partitioned between the granule and the soluble fraction (SSI, Li *et al.*, 1999a, SSII, Li *et al.*, 1999b) and a fourth form that is entirely located in the soluble fraction, SSIII (Cao *et al*, 2000, Li *et al.*, 1999b, Li *et al*, 2000). GBSS has been shown to be essential for amylose synthesis (Shure *et al.*, 1983), and mutations in SSII and SSIII have been shown to alter amylopectin structure (Gao *et*

10   *al*, 1998, Craig *et al.*, 1998). The rice GBSS (*waxy*) gene sequence has been described (Wang et al., 1990), and expression inhibited by antisense methods (Terada et al., 2000). The waxy gene is expressed in endosperm and pollen but not in other rice organs (Hirano and Sano, 2000).

15   Two main classes of SBEs are known in plants, SBEI and SBEII. SBEII can further be categorized into two types in cereals, SBEIIa and SBEIIb (Boyer and Preiss, 1978; Gao et al., 1996; Fisher et al., 1996; Hedman and Boyer, 1982; Mizuno *et al.*, 1992; Sun *et al.*, 1997; Sun et al., 1998). Additional forms of SBEs are also reported in some cereals, the putative 149kDa SBEI from wheat (Baga et al., 2000) and the 50/51kDa SBE from barley

20   (Sun et al., 1996). Genomic and cDNA sequences have been characterized for rice (Nakamura and Yamanouchi, 1992; Mizuno et al, 1992; Mizuno et al, 1993; Mizuno et al, 2001), maize (Baba et al., 1991; Fisher et al., 1993; Gao et al., 1997) wheat (Repellin et al., 1997; Nair et al., 1997; Rahman et al., 1997) and other cereals. Sequence alignment reveals a high degree of sequence similarity at both the nucleotide and amino acid levels and

25   allows the grouping into the SBEI, SBEIIa and SBEIIb classes. SBEIIa and SBEIIb generally exhibit around 80% sequence identity to each other, particularly in the central regions of the genes.

SBEIIa, SBEIIb and SBEI may also be distinguished by their expression patterns, both

30   temporal and spatial, in endosperm and in other tissues. SBEI is expressed from mid-endosperm development onwards in wheat and maize (Morell et al., 1997). In contrast, SBEIIa and SBEIIb are expressed from an early stage of endosperm development. In maize, SBEIIb is the predominant form in the endosperm whereas SBEIIa is present at high expression levels in the leaf (Gao et al., 1997). In rice, SBEIIa and SBEIIb are found

35   in the endosperm in approximately equal amounts (Yamanouchi and Nakamura, 1992). However, there were differences in timing and tissues of expression. SBEIIa is expressed

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at an earlier stage of seed development, being detected at 3 days after flowering (DAF), and was expressed in leaves, while SBEIIb was not detectable at 3 DAF and was most abundant in developing seeds at 7-10 DAF and was not expressed in leaves (Mizuno et al., 2001). In wheat endosperm, SBEI (Morell *et al*, 1997) is found exclusively in the soluble fraction, while SBEIIa and SBEIIb are found in both soluble and starch-granule associated fractions (Rahman et al., 1995).

Two types of debranching enzymes are present in higher plants and are defined on the basis of their substrate specificities, isoamylase type debranching enzymes, and pullulanase type debranching enzymes (Myers *et al.*, 2000). Sugary-1 mutations in maize and rice are associated with deficiency of both debranching enzymes (James *et al.*, 1995, Kubo *et al.*, 1999) however the causal mutation maps to the same location as the isoamylase-type debranching enzyme gene. In rice, antisense inhibition of isoamylase altered the structure of amylopectin and starch properties (Fujita et al., 2003), showing that isoamylase was required for amylopectin biosynthesis.

Representative starch branching enzyme genes that have been cloned from cereals are listed in Table 1.

Table 1. Starch branching enzyme genes characterized from cereals including rice.

Species	SBE isoform	Type of clone	Accession No.	Reference
Rice	SBEI	cDNA	D10752	Nakamura and Yamanouchi, 1992
	SBEI	genomic	D10838	Kawasaki et al., 1993
	RBE3	cDNA	D16201	Mizuno et al., 1993
	RBE4	cDNA	AB023498	Mizuno et al., 2001
Maize	SBEI	cDNA	U17897	Fisher et al., 1995
		genomic	AF072724	Kim et al., 1998a
	SBEIIb	cDNA	L08065	Fisher et al., 1993
		genomic	AF072725	Kim et al., 1998
	SBEIIa	cDNA	U65948	Gao et al., 1997
Wheat	SBEII	cDNA	Y11282	Nair et al., 1997
	SBEI	cDNA and genomic	AJ237897 SBEI gene) AF002821 (SBEI pseudogene AF076680 (SBEI gene) AF076679 (SBEI cDNA)	Baga et al., 1999 Rahman et al., 1997, Rahman et al., 1999
	SBEI	cDNA	Y12320	Repellin et al., 1997
	SBEIIa	cDNA and genomic	AF338432 (cDNA) AF338431 (gene)	Rahman et al., 2001
	SBEIIb	cDNA and genomic		WO 01/62934
	SBEIIb	cDNA		WO 00/15810
Barley	SBEIIa and SBEIIb	cDNA and genomic	AF064563 (SBEIIb gene) AF064561 (SBEIIb cDNA) AF064562 (SBEIIa gene) AF064560 (SBEIIa cDNA)	Sun et al., 1998

In maize and rice, high amylose phenotypes have been shown to result from lesions in the

5   *SBEIIb* gene, also known as the *amylose extender* (*ae*) gene (Boyer and Preiss, 1981,
Mizuno *et al.*, 1993; Nishi et al., 2001). In these *SBEIIb* mutants, endosperm starch grains
showed an abnormal morphology, amylose content was significantly elevated, the branch
frequency of the residual amylopectin was reduced and the proportion of short chains
(<DP17, especially DP8-12) was lower. Moreover, the gelatinisation temperature of the

10   starch was increased. In addition, there was a significant pool of material that was defined
as "intermediate" between amylose and amylopectin (Boyer *et al.*, 1980, Takeda, *et al.*,
1993b). In rice, inactivation of SBEIIb led to an amylose content of about 25% compared
to wild-type rice which has about 18% amylose (Nishi et al., 2001).

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In contrast, maize plants mutant in the *SBEIIa* gene due a mutator (Mu) insertional element
and consequently lacking in SBEIIa protein expression were indistinguishable from wild-
type plants in the branching of endosperm starch (Blauth *et al.*, 2001), although they were
altered in leaf starch. Similarly, rice plants deficient in SBEIIa activity exhibited no

5   significant change in the amylopectin chain profile in endosperm (Nakamura 2002). In
both maize and rice, the *SBEIIa* and *SBEIIb* genes are not linked in the genome.

Very high amylose varieties of maize have been known for some time. LAPS (low
amylopectin starch) maize which contains very high amylose content (>90%) was achieved

10   by a considerable reduction in the SBEI activity together with an almost complete
inactivation of SBEII activity (Sidebottom et al., 1998).

In potato, down regulation of the main SBE in tubers (SBE B, equivalent to SBEI) by
antisense methods resulted in some novel starch characteristics but did not alter the

15   amylose content (Safford et al., 1998). Antisense inhibition of the less abundant form of
SBE (SBE A, analogous to SBEII in cereals) resulted in a moderate increase in amylose
content to 38% (Jobling et al., 1999). However, the down regulation of both *SBEII* and
SBEI gave much greater increases in the relative amylose content, to 60-89%, than the
down-regulation of *SBEII* alone (Schwall *et al.*, 2000).

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In wheat, a mutant entirely lacking the SGP-1 (SSII) protein was altered in amylopectin
structure and had deformed starch granules and an elevated amylose content to about 30-
37 % of the starch, which was an increase of about 8% over the wild-type level (Yamamori
et al., 2000). Amylose was measured by colorimetric measurement, amperometric titration

25   (both for iodine binding) and a concanavalin A method. Starch from the SSII null mutant
exhibited a decreased gelatinisation temperature compared to starch from an equivalent,
non-mutant plant. Starch content of the grain was reduced from 60% in the wild-type to
below 50%.

30   In maize, the *dull1* mutation causes decreased starch content and increased amylose levels
in endosperm, with the extent of the change depended on the genetic background, and
increased degree of branching in the remaining amylopectin (Shannon and Garwood,
1984). The gene corresponding to the mutation was identified and isolated by a
transposon-tagging strategy using the transposon mutator (Mu) and shown to encode the

35   enzyme designated starch synthase II (SSII) (Gao et al., 1998). The enzyme is now
recognized as a member of the SSIII family in cereals (Li et al., 2003). Mutant endosperm

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had reduced levels of SBEIIa activity associated with the *dull1* mutation. It is not known if these findings are relevant to other cereals, for example rice.

Lines of barley having an elevated proportion of amylose in grain starch have been
5   identified. These include High Amylose Glacier (AC38) which has a relative amylose content of about 45%, and chemically induced mutations in the *SSIIa* gene of barley which raised levels of amylose in kernel starch to about 65-70% (WO 02/37955 A1; Morell *et al.*, 2003). The starch showed reduced gelatinisation temperatures.

10   Rice (*Oryza sativa* L.) is the most important cereal crop in the developing world and is grown widely, particularly in Asia which produces about 90% of the world total.

Starch is widely used in the food, paper and chemical industries. The physical structure of starch can have an important impact on the nutritional and handling properties of starch for
15   food or non-food or industrial products. Certain characteristics can be taken as an indication of starch structure including the distribution of amylopectin chain length, the degree and type of crystallinity, and properties such as gelatinisation temperature, viscosity and swelling volume. Changes in amylopectin chain length may be an indicator of altered crystallinity, gelatinisation or retrogradation of the amylopectin.
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Starch composition, in particular the form called resistant starch which may be associated with high amylose content, has important implications for bowel health, in particular health of the large bowel. Accordingly, high amylose starches have been developed in certain grains such as maize and barley for use in foods as a means of promoting bowel health.
25   The beneficial effects of resistant starch result from the provision of a nutrient to the large bowel wherein the intestinal microflora are given an energy source which is fermented to form *inter alia* short chain fatty acids. These short chain fatty acids provide nutrients for the colonocytes, enhance the uptake of certain nutrients across the large bowel and promote physiological activity of the colon. Generally if resistant starches or other dietary
30   fiber is not provided the colon is metabolically relatively inactive.

Whilst chemically or otherwise modified starches can be utilised in foods that provide functionality not normally afforded by unmodified sources, such processing has a tendency to either alter other components of value or carry the perception of being
35   undesirable due to processes involved in modification. Therefore it is preferable to provide sources of constituents that can be used in unmodified form in foods.

Therefore, rice having starch with a proportion of amylose greater than 40% is unknown. Although high amylose maize and barley varieties are known, very high amylose rice is preferred for rice growing regions. Starch from such rice is relatively resistant to digestion and therefore very high amylose rice is expected to bring an important health benefit to a

5   substantial portion of the world population.

GENERAL

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood

10   that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

15   Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the

20   purposes of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description. The references mentioned herein are hereby

25   incorporated by reference in their entirety. Reference herein to prior art, including any one or more prior art documents, is not to be taken as an acknowledgment, or suggestion, that said prior art is common general knowledge in Australia or forms a part of the common general knowledge in Australia.

30   As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

The designation of nucleotide residues referred to herein are those recommended by the

35   IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents Thymidine.

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SUMMARY OF THE INVENTION

In a first aspect the invention might be said to reside in grain obtained from a rice plant, comprising starch, wherein the proportion of amylose in the starch of the grain is at least 40%. The grain preferably has reduced activities or levels of SBEIIa and SBEIIb, and in

5 one form this might be achieved by two or more genetic variations, wherein one genetic variation is selected from the group consisting of a) a mutation of an *SBEIIa* gene which inhibits SBEIIa expression and/or activity, and b) an introduced nucleic acid which inhibits SBEIIa expression and/or activity, and wherein a second genetic variation is selected from the group consisting of a) a mutation of an *SBEIIb* gene which inhibits SBEIIb expression

10 and/or activity, and b) an introduced nucleic acid which inhibits SBEIIb expression and/or activity.

The grain of in one form comprises a transgene which transgene may encode an antisense, co-suppression, ribozyme or duplex RNA molecule. Alternatively the grain may be non-

15 transgenic the inhibition results from chromosomal mutation or rearrangement. The grain may comprise a null mutation of the *SBEIIa* or *SBEIIb* gene.

The grain of claim of the first aspect may comprise reduced levels of SBEIIa and SBEIIb proteins and/or activities. In a specific form the grain may further comprising a reduced

20 level of SBEI protein and/or activity or may additionally or alternatively also comprise an altered level of a protein and/or enzyme activity selected from the group consisting of ADP glucose pyrophosphorylase, GBSS, SSI, SSII, SSIII, a debranching enzyme of an isoamylase type and a debranching enzyme of a pullulanase type. In a specific alternative form the grain comprises an altered level of GBSS protein and/or enzyme activity. In a

25 further optional form the grain is of an Indica variety or which comprises a Wx^a allele.

The proportion of amylose in the starch of the grain in a preferable form is at least 50%.

The grain is preferably non-shrunken and in a specific form has in a brown rice form an

30 average weight of at least about 25 mg and preferably also a starch content that is at least 90% of the starch content of equivalent, but unaltered, grain. Preferably also at least 50% of starch granules within the grain appear non-birefringent when observed under polarized light.

35 The invention also encompasses in a second aspect a rice plant capable of producing the grain of the first aspect of the invention.

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Third and fourth aspects of the invention relates to starch and starch granules extracted from the grain of the first aspect of the invention.

In a fifth aspect the invention relates to a product comprising flour or starch produced
5   from the grain of the first aspect of the invention. The product may include a blend of the flour or starch with flour or starch from another source. The product may be a food or a non-food product.

A sixth aspect of the invention encompasses a composition comprising the starch of the
10   third aspect and another food ingredient or water.

A seventh aspect of the invention might be said to reside in a method of producing a rice plant capable of producing grain, the grain having starch comprising at least 40% amylose, comprising the steps of a) introducing a genetic variation into a parent rice plant or seed;
15   and b) identifying a progeny plant of the parent rice plant or seed, wherein the starch of grain of the progeny plant comprises at least 40% amylose. Preferably the genetic variation leads to a reduction of the levels of SBEIIa and SBEIIb proteins and/or activities in the endosperm of the rice plant.

20   The progeny rice plant of the method preferably comprises two or more genetic variations, wherein one genetic variation is selected from the group consisting of a) a mutation of an *SBEIIa* gene which inhibits SBEIIa expression and/or activity, and b) an introduced nucleic acid which inhibits SBEIIa expression and/or activity, and and wherein a second genetic variation is selected from the group consisting of c) a
25   mutation of an *SBEIIb* gene which inhibits SBEIIb expression and/or activity, and d) an introduced nucleic acid which inhibits SBEIIb expression and/or activity.

The step of introducing the genetic variation may comprise introducing an exogenous nucleic acid. The exogenous nucleic acid may be introduced into a rice cell which is then
30   regenerated into a rice plant. The exogenous nucleic acid preferably encodes an inhibitor of SBEIIa and/or SBEIIb expression and/or activity, and the inhibitor may an antisense, co-suppression, ribozyme or duplex RNA molecule.

Alternatively the step of introducing the genetic variation may comprise mutagenesis of the
35   parent rice plant or seed with a chemical agent or radiation.

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The progeny rice plant may comprise a null mutation in *SBEIIa* and/or *SBEIIb*.

The step of introducing a genetic variation may additionally leads to a reduction in the level
of SBEI protein and/or activity.

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The progeny plant may identified on the basis of the amylose level in the grain starch or
on a reduction in the levels of SBEIIa and/or SBEIIb proteins and/or activities in the
endosperm of the progeny plant.

10   The method may further comprise the introduction of a *Wx^f* allele into the rice plant, which
may be introduced by crossing.

An eighth aspect of the invention might be said to reside in a method of producing a rice
plant having a reduced level of both SBEIIa and SBEIIb proteins and/or enzyme activities
15   in the endosperm which comprises: a) mutagenising seed having a reduced level of SBEIIa
protein and/or enzyme activity; or b) mutagenising seed having a reduced level of SBEIIb
protein and/or enzyme activity; or c) crossing a plant having a reduced level of SBEIIa
protein and/or enzyme activity with a plant having a reduced level of SBEIIb protein and/or
enzyme activity; and d) identifying a rice plant having reduced activity of both SBEIIa and
20   SBEIIb proteins and/or enzyme activities in the endosperm.

The step of identifying the rice plant may comprise screening a population of rice plants
with a molecular marker that is linked to the *SBEIIa* gene or *SBEIIb* gene of rice, and
identifying the plant on the basis of the presence or absence of a signal from the screening
25   with the linked molecular marker.

The step of identifying the rice plant may comprises the step of screening seed from a
population of rice plants with an antibody that binds the SBEIIa protein or SBEIIb protein
of rice, and identifying the plant on the basis of the presence or absence of antibody
30   binding.

The invention may also encompass a method of producing altered rice starch comprising
the step of extracting starch from the grain of the first aspect of the invention.

35   The invention may additionally encompass the use of two or more exogenous nucleic acid
molecules, at least one of which encodes an inhibitor of rice *SBEIIa* expression and/or

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activity and at least another of which encodes an inhibitor of rice *SBEIIb* expression and/or activity, to produce a rice plant which has reduced levels of SBEIIa and SBEIIb proteins and/or activities. The inhibitors may be selected from the group consisting of antisense molecules, co-suppression molecules, ribozymes, duplex RNA molecules and any

5   combination of these.

The invention also encompasses an isolated nucleic acid molecule which encodes an inhibitor of rice SBEIIa and an inhibitor of rice SBEIIb, which may be the same or a different molecule. The isolated vector may be a vector. It will be understood that the

10   invention encompasses a cell which comprises the isolated nucleic acid molecule, which preferably is a rice cell. The invention will also be understood to encompass a transgenic rice plant comprising the isolated nucleic acid molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

15   Figure 1.   Sequence of a cDNA encoding rice Starch Branching Enzyme
                       I gene (SBEI)- Genbank Accession No. D11082 (SEQ. ID.
                       No 1).

       Figure 2.   Sequence of a cDNA encoding rice Starch Branching Enzyme IIa
20   gene (SBEIIa)- Genbank Accession No. AB023498 (SEQ ID No
                       2).

       Figure 3.   Sequence of a cDNA encoding rice Starch Branching Enzyme IIb
                       gene (SBEIIb)- Genbank Accession No. D16201 (SEQ ID NO 3).
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       Figure 4.   Schematic diagram of plasmid pRint9_BC containing a 500bp
                       fragment of intron 9 of rice SBEI gene inserted into pBC SK-.

       Figure 5.   Schematic of duplex-RNA constructs. A. The order of the gene
30   elements used were promoter, SBEIIa or SBEIIb cDNA sequence
                       in sense orientation, intron (Rint9), SBEIIa or SBEIIb cDNA
                       sequence in antisense orientation, and transcription
                       terminator/polyadenylation sequence. The transcript of the ds-
                       SBEIIa and ds-SBEIIb genes forms a "hairpin" RNA structure
35   with a double-stranded region formed by hybridization between the
                       sense and antisense sequences. The intron sequence bordered by

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the GT and AG nucleotides is spliced out. A schematic of
pRBEI.IR (pBC SK-(sense/Rint9/antisense)) is also shown.
Corresponding constructs pRBEIIa.IR and pRBEIIb.IR were also
made.

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Figure 6.   Schematic of plasmid vector pBx17casNOT is shown.

Figure 7.   Comparison of the rice SBEIIa (SEQ ID NO 2) and rice SBEIIb
                 (SEQ ID NO 3) cDNA sequences. The upper sequence
10   (uppercase) is from SBEIIb, the lower sequence (lowercase) from
                 SBEIIa. The 5' and 3' terminal sequences are not shown as they
                 do not have sufficient degree of identity.

Figure 8.   BLAST output from Gene Silencing program using SBEIIa,
15   SBEIIb and SBEI 3' sequences used in gene silencing constructs.

DETAILED DESCRIPTION OF THE INVENTION
Method of producing a rice plant
In an aspect, the invention provides a method of producing a rice plant having altered
20   starch in its grain, in particular increasing the relative proportion of amylose in the starch
to at least 40%. The proportion of amylose in the starch as defined herein is on a
weight/weight (w/w) basis, i.e. the weight of amylose as a percentage of the weight of
starch. The proportion of amylose in the starch is preferably at least 45%, 50%, 55% or
60% and even more preferably at least 65%, 70% or 75%. Ordinarily in rice, the
25   proportion of amylose in starch ranges from about 0 to 35%. The method may include
reducing the level of both starch branching enzyme IIa (SBEIIa) and starch branching
enzyme IIb (SBEIIb) protein or enzyme activity in the endosperm of rice. The reduction in
the proteins or activities may be by at least 40% or preferably by at least 60% compared to
the corresponding levels of protein or activity in the endosperm of unmodified rice, more
30   preferably by at least 75%, and even more preferably by at least 90% or 95%. One or
both of the proteins may be undetectable in the rice endosperm. The method may comprise
the alteration of the expression of the *SBEIIa* and *SBEIIb* genes of rice, or it may comprise
the mutation of the *SBEIIa* and *SBEIIb* genes in rice, or a combination of these, whereby
both the SBEIIa and SBEIIb activities in endosperm are reduced. The expression of either
35   or both genes may be inhibited by the introduction of a nucleic acid, for example a
transgene.

It would be readily apparent that the terms "increased", "decreased", "reduced" "altered" and the like as used herein are comparative terms that refer to a difference in the ~~plants or products of the invention in comparison with corresponding wild-type plants or~~

5   products, said wild type plants or products not being modified according to the invention.

Amylose is defined herein as including essentially linear molecules composed of α-1,4 linked glucosidic molecules and amylose-like long-chain amylopectin (sometimes referred to as "intermediate material", Takeda et al., 1993b; Fergason, 1994). Amylose content

10   may be determined by any of the methods known in the art including size exclusion HPLC, for example in 90% (w/v) DMSO, concanavalin A methods (Megazyme Int, Ireland), or preferably by iodometric methods, for example as described in Example 1. The HPLC method may involve debranching of the starch (Batey and Curtin, 1996) or not involve debranching. From the grain weight and amylose content, the amount of amylose

15   deposited per grain can be calculated and compared for transgenic and control lines.

The method may comprise the step of determining the activities of SBEIIa and/or SBEIIb, preferably both, in rice endosperm. This may be done by measuring the levels of the protein, for example by immunodetection, or the levels of their corresponding mRNAs by

20   methods well known in the art such as Northern blot hybridization analysis, slot-blot hybridization, RNAse protection assays, microarray analysis or reverse transcription polymerase chain reaction (RT-PCR). The method may further comprise the step of screening for a rice plant or grain having reduced SBEIIa and/or SBEIIb activities in its endosperm, or selecting or identifying such a plant or grain. The screening/selection step

25   may be based on the reduced level of the SBEIIa and/or SBEIIb activity or protein, or it may be based on the phenotype of the grain of the rice plant such as an increased proportion of amylose or decreased proportion of amylopectin or a visual phenotype, for example shrunken grain.

30   SBE activity may be measured by enzyme assay, for example by the phosphorylase stimulation assay (Boyer and Preiss, 1978). This assay measures the stimulation by SBE of the incorporation of glucose 1-phosphate into methanol-insoluble polymer (α-D-glucan) by phosphorylase a. SBE activity can be measured by the iodine stain assay, which measures the decrease in the absorbency of a glucan-polyiodine complex resulting

35   from branching of glucan polymers. SBE activity can also be assayed by the branch linkage assay which measures the generation of reducing ends from reduced amylose as

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substrate, following isoamylase digestion (Takeda et al., 1993a). Preferably, the activity is measured in the absence of SBEI activity. Isoforms of SBE show different substrate specificities, for example SBEI exhibits higher activity in branching amylose, while SBEIIa and SBEIIb show higher rates of branching with an amylopectin substrate. The

5   isoforms may also be distinguished on the basis of the length of the glucan chain that is transferred. SBE protein may also be measured by using specific antibodies such as those described herein. In a preferred embodiment, SBEIIa and SBEIIb protein levels are measured by immunological methods such as Western blotting or ELISA assay using specific antibodies raised to polypeptide fragments corresponding to the N-terminal amino

10   acid sequences of rice SBEIIa and SBEIIb. The SBEII activity may be measured during grain development in the developing endosperm, or alternatively in the mature grain where the protein is still present in equivalent, but unaltered, grain and can be assayed by immunological methods.

15   In a further aspect, the invention provides a method of altering, preferably reducing, the activity of a third starch biosynthesis enzyme in rice, in combination with the reduction in activity of SBEIIa and SBEIIb, such that the proportion of amylose in the starch of the grain is at least 40%. Preferably, SBEI activity in the endosperm is also reduced. Other starch biosynthesis enzymatic activities that may be altered in combination with SBEIIa

20   and SBEIIb are: SSI, SSII, SSIII. Starch debranching enzymes may also be altered, for example the activity of isoamylase or pullulanase. The third starch biosynthetic enzyme activity may be increased or decreased, preferably decreased, by at least 40% compared to the activity in unmodified rice, preferably at least 60% or 80% and more preferably at least 90%.

25

In a further embodiment, the activities of starch biosynthesis enzymes may be altered in the plant in tissues other than endosperm, for example the activity of SBEI or SBEII, preferably SBEIIa, may be increased in leaves to compensate for some loss of activity caused by a genetic variation in the plant that leads to loss of SBEIIa activity in the

30   endosperm. This is particularly preferred when the genetic variation leads to reduction in SBEIIa activity not only in the endosperm but also in other tissues, in particular the leaves. It would be appreciated that such compensation of activity in tissues other than the endosperm would, for example, be from an enzyme coding region under the control of a promoter that is not expressed in the endosperm. This may be a promoter from a

35   photosynthesis-related gene such as *rbcS*. Alternatively, starch synthesis in the endosperm may be further improved by the overexpression of one or more starch biosynthetic

enzymes in combination with a reduction in SBEIIa and SBEIIb activities in the endosperm. Genes encoding such enzymes may be from any of a variety of sources, for example from bacterial or other sources other than rice, and may be modified to alter the catalytic properties, for example alteration of the temperature dependence of the enzymes

5   (WO94/09144).

The high amylose phenotype may be achieved by partial or full disruption to the expression of the *SBEIIa* and *SBEIIb* genes. The method of the invention may comprise the step of screening or identifying or selecting a rice plant or grain which has a null

10   mutation in the SBEIIa and/or SBEIIb genes. A "null mutation" is defined herein as a mutation that leads to the lack of detectable protein or enzyme activity in the plant tissue of interest, preferably the endosperm. The screening/identifying step may therefore comprise a screen at the gene level, for example a screen for deletions in the gene encoding SBEIIa and/or SBEIIb, or at the level of expression of the gene of interest. The extent to which the

15   genes are inhibited will in some degree determine the characteristics of the starch made in the rice grain. Screening for deletions may conveniently be carried out by PCR amplification methods using primers designed such that at least part of the amplification product spans at least part of the gene of interest. Any of a range of gel electrophoresis techniques carried out on the proteins extracted from the modified rice endosperm will

20   reveal the nature and extent of modification to the SBEIIa and SBEIIb activities. Modification may occur as a reduction in SBEIIa and/or SBEIIb activities or complete abolition of enzyme activity within the endosperm. To carry out these tests, starch may be extracted from the rice endosperm and the proteins therein analyzed. Techniques well known in the art such as SDS-PAGE and immunoblotting may be carried out on the

25   soluble and the starch granule fractions and identify the plants or grain where modifications have occurred to the SBEIIa and SBEIIb enzymes.

The method of the invention may comprise the introduction of a genetic variation into the rice plant or an ancestral rice plant or seed. The genetic variation may comprise a

30   transgene, as described below, or may be introduced by mutagenesis, for example by chemical mutagenesis or by radiation.

Rice plants

In a further aspect, the invention provides a rice plant capable of producing grain having a

35   proportion of amylose in the starch of at least 40%. A rice plant is defined herein as any plant of the species *Oryza sativa* L. The rice plant may be of any of the three recognised

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races of *O. sativa* L., namely Japonica (or sinica), Indica and Javanica and is preferably an
Indica variety. There are numerous cultivars or varieties in each race and all are included in
the plants of the invention. Preferred cultivars are those grown in Australia including, for
example, cultivars Amaroo, Ali Combo, Basmati, Bogan, Bombla, Doongara, Goolarah,

5   Illabong, Jarrah, Koshihikari, Kyeema, Langi, Millin, Namage, Opus, Pelde. The
proportion of amylose is preferably at least 45%, 50%, 55%, 60%, 65%, 70% or 75%. The
rice plant comprises at least one genetic variation that inhibits SBEIIa and/or SBEIIb
expression and/or activities in the endosperm. The genetic variation may be any genetic
variation or combination of genetic variations that leads to a reduction in both SBEIIa and

10   SBEIIb activities and/or proteins in rice endosperm, such as mutations in the *SBEIIa* and
SBEIIb genes, an introduced nucleic acid such as a gene encoding an antisense, enhanced
antisense, co-suppression, ribozyme, duplex RNA or similar molecule that inhibits SBEIIa
and/or SBEIIb expression or activity, and a combination of the above. The genetic
variation is preferably a null mutation. Plants having reduced SBEIIa and SBEIIb activities

15   may be produced by crossing a plant reduced for SBEIIa with a plant reduced for SBEIIb,
or by introducing a transgene encoding a molecule that inhibits expression of both *SBEIIa*
and *SBEIIb* genes. In a preferred embodiment, the rice plant has null mutations in both
SBEIIa and *SBEIIb*.

20   The invention also provides rice plants with reduced levels of both SBEIIa and SBEIIb
activities in the endosperm during at least some of the development of the grain, the rice
plant being capable of bearing grain having starch comprising an increased proportion of
amylose compared to starch extracted from equivalent, but unaltered, plant. Preferably, the
levels of SBEIIa and SBEIIb are reduced in the endosperm by at least 50%, more

25   preferably by at least 75% and most preferably by at least 90% or 95% compared to the
wild-type. The term "wild-type" has its normal meaning in the field of genetics and
includes rice cultivars or genotypes which are not modified as taught herein.

The invention also provides progeny plants and grain which have the desired
30   characteristics of the parent rice plants, in genotype and/or phenotype. The invention also
extends to any propagating material of the rice plants that can be used to produce the
plants with the desired characteristics, such as cultured tissue or cells.

The altered rice plants of the invention may be crossed with plants containing a more
35   desirable genetic background, and therefore the invention includes the genetic variation(s)
in other genetic backgrounds. After the initial crossing, a suitable number of backcrosses

may be carried out to remove the less desirable background. The desired genetic background may include a suitable combination of genes providing commercial yield and other characteristics such as agronomic performance or abiotic stress resistance. The genetic background might also include other altered starch biosynthesis or modification

5   genes, for example genes from other rice lines that have a shrunken endosperm where the causal gene is not known.

In a preferred embodiment, the rice plant comprises the Wx^a allele of the waxy gene. This allele is found mostly in Indica varieties of rice, while the Wx^b allele is found mostly in

10   Japonica varieties. The Wx^b allele carries a substitution mutation (GT to TT) at the 5' splice site of the first intron of the waxy gene, resulting in lower waxy gene expression and therefore lower GBSS activity and lower amylose levels than in corresponding plants containing the Wx^a allele (Isshiki et al., 1998; Hirano et al., 1998; Frances et al., 1998).

15   The plants or grain therefrom may be transgenic or non-transgenic.

Grain

The invention also provides rice grain comprising an altered starch compared to starch extracted from an equivalent, but unaltered, rice plant. Grain is defined herein as essentially

20   mature grain. This includes grain as harvested in a commercial setting. At harvest, rice grain may be in the form of rough rice, which includes the hull, or "brown rice" where the hull is removed. Only the brown rice fraction is edible. Brown rice consists of the outer layers of pericarp, seed-coat and nucellus, the germ (embryo) and the endosperm. The endosperm as defined herein consists of the aleurone layer and the endosperm proper,

25   consisting of the subaleurone layer and the starchy or inner endosperm. Brown rice may be milled by abrasive or friction milling to remove the pericarp, seed-coat, testa, aleurone layer and embryo to yield milled rice, which essentially comprises the starchy endosperm. Milling results in loss of the constituents of the seed-coat and aleurone including some of the fat, protein, fiber, minerals and vitamins including thiamine, riboflavin, niacin and a-

30   tocopherol. The carbohydrate content, mainly starch, is higher in milled rice than in brown rice. Milled wild-type rice grain contains about 77-89% carbohydrate, 6.3-7.1% protein, 1.5-1.7% lipid including both starch-bound and non-starch forms, 0.3-0.8% minerals, 0.3-0.5% crude fibre, 0.7-2.3% neutral detergent fiber and may contain moisture of about 10-15%. "Rice grain" or simply "rice" as defined herein includes rough rice, brown rice

35   and milled rice, and is preferably milled rice.

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The rice grain of the invention comprises at least one genetic variation as defined herein
for the rice plant from which the grain is derived. The genetic variation(s) lead to a
reduction in SBEIIa and SBEIIb activities and/or proteins during development of the
endosperm of the rice grain. The grain comprises an increased proportion of amylose (as

5   a percentage of total starch) and a reduced proportion of amylopectin compared to grain
from the equivalent, but unaltered, plant.   Starch is the major constituent of milled rice,
comprising about 90% of the dry matter. The amylose content of the endosperm starch of
rice which is not modified according to the invention is in the range 0-37%, depending on
the genotype. Based on iodometric (colorimetric) assays which measure the "apparent

10   amylose content", milled rice is classified as waxy (1-2% amylose), very low amylose (2-
12%), low amylose (12-20%), intermediate amylose (20-25%) and high amylose (25-
33%), (Juliano, 1979, 1985). Recent studies using HPLC assays showed that the
maximum true amylose content is about 20% and that additional iodine binding is due to
the long linear chains in amylopectin (Takeda et al., 1987). The "amylose content" or

15   "apparent amylose content" as defined herein is determined by an iodometric method, as
known to those skilled in the art, for example the spectrophotometric method described by
Morrison and Laignelet (1983). It will be appreciated that other methods such as the high-
performance liquid chromatography (HPLC, for example, Batey and Curtin, 1996)
methods which assay only the "true amylose" may underestimate the amylose content as

20   defined herein.

The grain of the invention has starch comprising at least 40% (w/w) amylose. The
proportion of amylose is preferably at least 45%, 50% or 55% of the total starch, more
preferably at least 60% and even more preferably at least 65%, 70%, or 75%. In a

25   preferred embodiment, the grain is non-transgenic and its starch comprises at least 40%
amylose. Alternatively, the grain has reduced levels of SBEIIa and SBEIIb proteins and its
starch has at least 40% amylose. Increased amylose levels may be evidenced by abnormal
starch granule morphology or loss of birefringence of the granules when observed under a
light microscope or other methods known in the art.

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In a preferred embodiment, the rice is of an Indica variety or comprises the Wx^a allele of
the *waxy* gene.

The grain may comprise starch that has altered physical characteristics, for example

35   increased or decreased gelatinisation temperatures and/or altered swelling characteristics
during and following gelatinisation.

The grain may be shrunken or non-shrunken, preferably having a non-shrunken phenotype. "Non-shrunken" as used herein is defined as where the majority of grains, preferably at least 90% of the individual grains, show a plump or fully-filled phenotype.

5   This is usually associated with a normal or near normal level of starch accumulation. In contrast, a "shrunken" phenotype as used herein refers to the majority of grains, particularly at least 90% of the grains, having reduced starch accumulation. Slightly shrunken grain refers to a reduction in average starch content of at least 30%, moderately shrunken grain refers to a reduction in average starch content of at least 50%, and highly

10 shrunken grain refers to a reduction in average starch content of at least 70%. Shrunkenness may also be measured by the relative starch content, as a percentage of mature grain weight. The parameters for wild-type brown rice grain size and shape may be defined as: extra long, >7.50 mm; long, 6.61 to 7.50 mm; medium, 5.51 to 6.60 mm; and short, <5.50 mm. Grain shape may be characterized based on length-to-width ratio and is

15 defined as slender, >3.0; medium, 2.1 to 3.0; bold 1.1 to 2.0; and round, < 1.0. Each of these characteristics may be altered in the rice grain of the invention.

The invention also provides flour, meal or other products produced from the grain. These may be unprocessed or processed, for example by fractionation or bleaching. The

20 invention further provides rice grain useful for food production obtained from the rice plant of the invention. Additionally the invention encompasses grain that has been processed in other ways, so that the grain may have been milled, ground, rolled, pearled, kibbled or cracked, or boiled.

25 *Starch*

In another aspect, the invention provides starch granules or starch obtained from the grain of the rice plant as described above, having an increased proportion of amylose and a reduced proportion of amylopectin. The plant from which the grain was obtained has reduced levels of SBEIIa and SBEIIb activities in the endosperm and more preferably the

30 activity of SBEI is also reduced. In another aspect, the invention provides starch granules or starch obtained from the grain of the rice plant, comprising at least 40% amylose, preferably at least 45%, 50%, 55% or 60% amylose, and even more preferably at least 65%, 70%, or 75% amylose. Purified starch may be obtained from grain by a milling process, for example a wet milling process, which involves the separation of the starch

35 from protein, oil and fiber. The initial product of the milling process is a mixture or composition of starch granules, and the invention therefore encompasses such granules.

Starch granules of wild-type rice are polyhedral in shape and mainly 3 to 9μm in size,
averaging about 5μm with unimodal distribution in size. Protein occurs mainly in the form
of spherical protein bodies 0.5 to 4 μm in size throughout the endosperm.

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The starch may have an increased or reduced gelatinisation temperature, preferably an
increased gelatinisation temperature. The gelatinisation temperature, in particular the
temperature of onset of the first peak or the temperature for the apex of the first peak, may
be elevated by at least 3°C, preferably at least 5°C or more preferably at least 7°C as
10   measured by DSC compared to starch extracted from a similar, but unaltered grain. The
starch may comprise an elevated level of resistant starch, with an altered structure indicated
by specific physical characteristics including one or more of the group consisting of
physical inaccessibility to digestive enzymes which may be by reason of having altered
starch granule morphology, the presence of appreciable starch associated lipid, altered
15   crystallinity, and altered amylopectin chain length distribution. The high proportion of
amylose also contributes to the level of resistant starch.

The invention also provides starch from grain of the exemplified rice plant comprising
increased amounts of dietary fibre, preferably in combination with an elevated level of
20   resistant starch. This increase is also at least in part a result of the high relative level of
amylose.

Methods of reducing gene activity: Transgenes
The activity of *SBEIIa*, *SBEIIb* or other starch biosynthesis or modification genes are
25   preferably altered by introducing a genetic variation into the rice plant. This may be by
means of the introduction of a transgene into the rice plant. A "genetic variation" means
any alteration in the genome which, in this context, leads to a reduction in the activities of
SBEIIa and SBEIIb and optionally other starch biosynthesis or modification genes, and
includes mutations such as point mutations, substitutions, inversions, duplications,
30   translocations and preferably deletions, as well as introduction of transgenes into genes or
control elements. In a preferred embodiment, the genetic variation is a null mutation, for
example as a consequence of an inversion, duplication, translocation, deletion, frameshift
or RNA splicing mutation. A "transgene" as referred to herein has the normal meaning in
the art of biotechnology and includes a genetic sequence which has been produced or
35   altered by recombinant DNA or RNA technology and which has been introduced into the
organism or cell of interest. The transgene may include genetic sequence derived from the

organism or cell, for example an antisense sequence. In a preferred embodiment, the transgene comprises a nucleotide sequence having at least 19 consecutive nucleotides having at least 94% identity with at least 19 consecutive nucleotides of the complement of the rice SBEIIa sequence defined herein or the rice SBEIIb sequence defined herein. The

5   transgene typically includes an exogenous nucleic acid which is not derived from rice. "Transgenic" refers to the rice plant or grain or cell containing a transgene. "Non-transgenic" refers to the absence of any transgene in the genome of the rice plant, grain or cell. A transgene is preferably integrated into the genome of the rice plant, grain or cell, for stable inheritance.

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Reference herein to a "gene" including an SBEIIa, SBEIIb or other starch biosynthetic gene or genes encoding antisense, enhanced antisense, co-suppression, ribozyme, duplex RNA molecules or the like is to be taken in its broadest context and includes a genomic gene as well as mRNA or cDNA corresponding to the coding regions (i.e. exons) of the

15   gene, if they are present, transcribed but not translated sequences, and regulatory regions including promoters and transcription terminators/polyadenylation sequences. The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product. Preferred genes are derived from naturally occurring SBEIIa, SBEIIb or starch biosynthetic genes by standard recombinant techniques. Generally, a gene may

20   be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or additions. Nucleotide insertional derivatives of such genes include 5' and 3' terminal fusions as well as intra-sequence insertions of single or multiple nucleotides. Insertional nucleotide sequence variants are those in which one or more nucleotides are introduced into a predetermined site in the nucleotide sequence, although random insertion

25   is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more nucleotides from the sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide inserted in its place. Such a substitution may be "silent" in that the substitution does not change the amino acid defined by the codon.

30   Alternatively, substituents are designed to alter one amino acid for another similar acting amino acid. Typical conservative substitutions are those made in accordance with the following:

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Suitable residues for amino acid substitutions

	Original Residue	Exemplary Substitutions
	Ala	Ser
5	Arg	Lys
	Asn	Gln; His
	Asp	Glu
	Cys	Ser
	Gln	Asn
10	Glu	Asp
	Gly	Ala
	His	Asn; Gln
	Ile	Leu; Val
	Leu	Ile; Val
15	Lys	Arg; Gln; Glu
	Met	Leu; Ile
	Phe	Met; Leu; Tyr
	Ser	Thr
	Thr	Ser
20	Trp	Tyr
	Tyr	Trp; Phe
	Val	Ile; Leu

25   Those skilled in the art will be aware that expression of a gene, or a complementary sequence thereto, in a cell, requires said gene to be placed in operable connection with a promoter sequence. The choice of promoter for the present purpose may vary depending upon the level of expression required and/or the tissue, organ or cell in which expression is to occur, particularly endosperm specific promoters.

30   Placing a nucleic acid molecule under the regulatory control of a promoter sequence means positioning said molecule such that expression is controlled by the promoter sequence. A promoter is usually, but not necessarily, positioned upstream, or at the 5'-end, of the nucleic acid molecule it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription

35   of the gene. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position the promoter at a distance from the gene transcription

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start site that is approximately the same as the distance between that promoter and the gene
it controls in its natural setting (i.e., the gene from which the promoter is derived). As is
known in the art, some variation in this distance can be accommodated without loss of
promoter function. Similarly, the preferred positioning of a regulatory sequence element

5   with respect to a heterologous gene to be placed under its control is defined by the
positioning of the element in its natural setting (i.e., the gene from which it is derived).
Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in gene constructs of the present invention include

10   promoters derived from the genes of viruses, yeast, moulds, bacteria, insects, birds,
mammals and plants, preferably those capable of functioning in plant cells, more
preferably those capable of being expressed in the endosperm of rice. The promoter may
regulate expression constitutively, or differentially, with respect to the tissue in which
expression occurs. Alternatively, expression may be differential with respect to the

15   developmental stage at which expression occurs, or in response to external stimuli such as
physiological stresses, or temperature.

The method of reducing SBEIIa or other starch biosynthetic gene activity may comprise
the step of introducing a transgene into a regenerable cell of rice and regenerating a

20   transgenic rice plant from the transformed cell. The branching enzymes involved in
synthesis of amylopectin include SBEI, SBEIIa and SBEIIb and the transgene(s) may
inactivate more than one of these genes. Moreover, the inactivation of SBEIIb and/or
SBEI may be direct, in that the transgene (e.g. encoding duplex RNA, antisense, or
ribozyme RNA, see below) directly targets the *SBEIIb* or *SBEI* gene expression, or it may

25   indirectly result in the reduction in the expression of *SBEIIb* or *SBEI*. For example, the
transgene RNA may target only the *SBEIIa* gene/RNA in terms of sequence identity or
basepairing but also result in reduction of SBEIIb or SBEI activity by altering protein
stability or distribution in the endosperm. Additional forms of the present invention reside
in the combination of reduced activity of SBEIIa and SBEIIb and an alteration of one or

30   more other amylopectin synthesis enzymes, which enzymes may include SSI, SSII, SSIII,
and debranching enzymes such as isoamylase or pullulanase. Expression of any or all of
these may be altered by introduction of a transgene. In a particular embodiment, ADP-
glucose pyrophosphorylase (ADGP) is overexpressed in the rice plants, which has been
shown to enhance yield and growth (Smidansky et al. 2003).

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Several DNA sequences are known for amylopectin synthesis genes in rice, any of which can be the basis for designing transgenes for inactivation of the genes in rice. These include rice cDNAs *SBEIIa* (GenBank accession number E14723, Japanese patent application No. JP1998004970), *SBEIIb* (D16201, Mizuno et al., 1993) and *SBEI*

5   (D11082, Mizuno et al 1992; D10752, Nakamura and Yamanouchi, 1992). The *SBEI* gene of rice is described in Rahman et al., (1997) and Rahman et al., (1999), or Accession No. D10838, Kawasaki et al., 1993). Further gene sequences may be obtained from the following websites: http://www.ncbi.nlm.nih.gov/; http://www.tigr.org; http://www.gramene.org/about/index.html.

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Homologues of *SBEIIa*, *SBEIIb* or other amylopectin synthesising genes from wheat, barley, maize or other closely related species can also be used to modify gene expression levels in rice. Such genes or fragments thereof can be obtained by methods well known in the art, including PCR amplification or hybridization to labeled probes. The region(s) of

15 the homologues used in preparing the transgene construct should have at least 85% identity to the corresponding rice gene, preferably at least 90% and even more preferably 95-100% identity in the appropriate region. It is also preferred that the transgene specifically target the amylopectin synthesis genes expressed in the endosperm of rice and have less or minimal effect on amylopectin synthesis elsewhere in the plant. This may be

20 achieved by use of suitable regulatory sequences such as endosperm-specific promoters in the transgene.

"Stringent hybridization conditions" as used herein means that hybridization will generally occur if there is at least 90% and preferably at least 95% sequence identity

25 between the probe and the target sequence. Examples of stringent hybridization conditions are overnight incubation in a solution comprising 50% formamide, 5 x SSC (1xSSC = 150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured sheared carrier DNA such as salmon sperm DNA, followed by washing the hybridization support in 0.1 x SSC

30 at approximately 65°C. Other hybridization and wash conditions are well known and are exemplified in Sambrook et al, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly chapter11.

Antisense

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Known genetic engineering or transgenic approaches to altering, in particular specifically reducing, gene activity in plants are well known in the art. These methods of introducing genetic variation into the rice plant include the expression of a suitable antisense molecule that is complementary to the RNA of the target gene and can hybridize with it. Antisense

5   molecules are thought to interfere with the translation or processing or stability of the mRNA of the target gene, thereby inactivating its expression. Methods of devising antisense sequences are well known in the art and examples of these are can be found in United States Patent No. 5190131, European patent specification 0467349-A1, European patent specification 0223399-A1 and European patent specification 0240208, which are

10   incorporated herein by reference. The use of antisense techniques in plants has been reviewed by Bourque (1995) and Senior (1998). Bourque lists a large number of examples of how antisense sequences have been utilized in plant systems as a method of gene inactivation. She also states that attaining 100% inhibition of any enzyme activity may not be necessary as partial inhibition will more than likely result in measurable

15   change in the system. Senior (1998) states that antisense methods are now a very well established technique for manipulating gene expression.

Antisense molecules for rice *SBEIIa*, *SBEIIb*, *SBEI* or other amylopectin biosynthesis genes can be based on the rice mRNA sequences or based on homologies with DNA or

20   mRNA sequences derived from other species, for example barley. These antisense sequences may correspond to the structural genes or for sequences that effect control over the gene expression or splicing event. For example, the antisense sequence may correspond to the targeted coding region of the rice *SBEIIa* or other gene, or the 5'-untranslated region (UTR) or the 3'-UTR or combination of these. It may be

25   complementary in part to intron sequences, which may be spliced out during or after transcription, preferably only to exon sequences of the target gene. In view of the generally greater divergence of the UTRs, targeting these regions provides greater specificity of gene inhibition. The length of the antisense sequence should be at least 19 contiguous nucleotides, preferably at least 50 nucleotides, and more preferably at least 100,

30   200, 500 or 1000 nucleotides. The full-length sequence complementary to the entire gene transcript may be used. The length is most preferably 100-2000 nucleotides. The degree of homology of the antisense sequence to the targeted transcript should be at least 85%, preferably at least 90% and more preferably 95-100%. The antisense RNA molecule may of course comprise unrelated sequences which may function to stabilize the molecule.

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Cosuppression

Another molecular biological approach that may be used is co-suppression. The mechanism of co-suppression is not well understood but is thought to involve post-transcriptional gene silencing (PTGS) and in that regard may be very similar to many

5   examples of antisense suppression. It involves introducing an extra copy of a gene or a fragment thereof into a plant in the sense orientation with respect to a promoter for its expression. The size of the sense fragment, its correspondence to target gene regions, and its degree of homology to the target gene are as for the antisense sequences described above. In some instances the additional copy of the gene sequence interferes with the

10   expression of the target plant gene. Reference is made to Patent specification WO 97/20936 and European patent specification 0465572 for methods of implementing co-suppression approaches.

Double stranded RNA-mediated gene silencing

15   A further method that might be employed to introduce genetic variation into the rice plant is duplex or double stranded RNA mediated gene silencing. This method also involves PTGS. In this method a DNA is introduced that directs the synthesis of an at least partly double stranded RNA product(s) with homology to the target gene to be inactivated. The DNA therefore comprises both sense and antisense sequences that, when transcribed into

20   RNA, can hybridize to form the double-stranded RNA region. In a preferred embodiment, the sense and antisense sequences are separated by a spacer region that comprises an intron which, when transcribed into RNA, is spliced out. This arrangement has been shown to result in a higher efficiency of gene silencing (Smith et al., 2000). The double-stranded region may comprise one or two RNA molecules, transcribed from either one

25   DNA region or two. The presence of the double stranded molecule triggers a response from an endogenous plant system that destroys both the double stranded RNA and also the homologous RNA transcript from the target plant gene, efficiently reducing or eliminating the activity of the target gene. Reference is made to Australian Patent specification 99/292514-A and Patent specification WO 99/53050 for methods of

30   implementing this technique. The length of the sense and antisense sequences that hybridise should each be at least 19 contiguous nucleotides, preferably at least 30 or 50 nucleotides, and more preferably at least 100, 200, 500 or 1000 nucleotides. The full-length sequence corresponding to the entire gene transcript may be used. The lengths are most preferably 100-2000 nucleotides. The degree of homology of the sense and

35   antisense sequences to the targeted transcript should be at least 85%, preferably at least 90% and more preferably 95-100%. The RNA molecule may of course comprise

unrelated sequences which may function to stabilize the molecule. The RNA molecule may be expressed under the control of a RNA polymerase II or RNA polymerase III promoter. Examples of the latter include tRNA or snRNA promoters.

5   The antisense, cosuppression or double stranded RNA molecules may also comprise a largely double-stranded RNA region, preferably comprising a nuclear localization signal, as described in PCT/AU03/00292. In a preferred embodiment, the largely double-stranded region is derived from a PSTVd type viroid or comprises at least 35 CUG trinucleotide repeats.

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Ribozymes

Ribozymes may be used to introduce the genetic variation responsible for inactivation of the desired gene expression in rice. Ribozymes are RNA molecules with enzymatic or catalytic function that can cleave other RNA molecules at specific sites defined by one or

15   often two hybridizing sequences. The cleavage of the RNA inactivates the expression of the target gene. The ribozymes may also act as an antisense molecule, which may contribute to the gene inactivation. The ribozymes contain one or more catalytic domains, preferably of the hammerhead or hairpin type, between the hybridizing sequences. Other ribozyme motifs may be used including RNAseP, Group I or II introns, and hepatitis delta

20   virus types. Reference is made to European patent specification 0321201 and US Patent No. 6,221,661. The use of ribozymes to inactivate genes in transgenic plants has been demonstrated, for example by Wegener et al (1994).

Genetic constructs/vectors

25   The invention also provides isolated nucleic acid molecules including RNA and preferably DNA which encode the gene-inhibiting molecule. Preferably, the nucleic acid molecules encode the antisense, sense (co-suppression), double-stranded RNA or ribozyme molecules targeting the rice *SBEIIa* and/or *SBEIIb* gene sequences and effective in inactivating their expression in endosperm of rice grain. The invention also provides

30   genetic constructs comprising the isolated nucleic acid molecule, comprising one or more regulatory elements such as promoters, enhancers and transcription termination or polyadenylation sequences. Such elements are well known in the art. The genetic constructs may also comprise intron sequences that aid expression of the transgene in plants, particularly in monocotyledonous plants such as rice. The term "intron" is used in

35   its normal sense as meaning a genetic segment that is transcribed but does not encode protein and which is spliced out of an RNA before translation. Introns may be

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incorporated in a 5'-UTR or a coding region if the transgene encodes a translated product,
or anywhere in the transcribed region if it does not. In a particular embodiment, introns
that direct endosperm-specific expression such as the barley SBEII gene intron
(Ahlandsberg et al., 2002) are used.

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The invention further provides vectors, for example plasmid vectors, comprising the
genetic constructs. The term "vector" includes an expression vector, being capable of *in
vitro* or *in vivo* expression, and a transformation vector, capable of being transferred from
one cell or organism to another. The vectors comprise sequences that provide for

10   replication in cells, for example in prokaryotic cells such as *E. coli* or *Agrobacterium*.
Preferably, the vector is a binary vector comprising a T-DNA sequence, defined by at least
one T-DNA border sequence, that can be introduced into rice cells. The invention further
provides cells comprising the vectors, for example *Agrobacterium* or rice cells which may
be regenerable cells such as the cells of the scutellum of immature embryos or

15   embryogenic callus. Alternatively, the cells may be transformed rice cells comprising the
transgene.

Promoters/terminators

The transgene or other genetic construct of the invention may include a transcriptional

20   initiation region (promoter) that may provide for regulated or constitutive expression in the
endosperm of rice. The promoter may be tissue specific, conferring expression selectively
or exclusively in the endosperm. The promoter may be selected from either endosperm-
specific (such as High Molecular Weight Glutenin promoter, the rice SSI promoter, rice
SBEII promoter, rice GBSS promoter) or promoters not specific for the endosperm (such

25   as ubiquitin promoter or CaMV35S or enhanced 35S promoters). The promoter may be
modulated by factors such as temperature, light or stress. Ordinarily, the promoter would
be provided 5' of the genetic sequence to be expressed. The construct may also contain
other elements that enhance transcription such as the nos 3' or the ocs 3' polyadenylation
regions or transcription terminators. The regions of DNA illustrated will be incorporated

30   into vectors containing suitable selectable marker gene sequences and other elements, or
into vectors that are co-transformed with vectors containing these sequences.

Transformation methods for rice

Methods for transformation of rice, that is for introducing genetic variation into the plant

35   by introduction of an exogenous nucleic acid, are well known in the art, see for example,
Chan et al., 1993; Hiei et al., 1994; Zhang et al., 1997; Buchholz et al., 1998.

Transformation may be mediated by suitable *Agrobacterium* strains, or by biolistic methods, or by polyethylene glycol mediated uptake into rice protoplasts, or the like, as known in the art. Vectors carrying the desired nucleotide sequence or genetic construct and a selectable marker may be introduced into regenerable rice cells of tissue cultured

5   plants or explants, for example protoplasts or immature embryos or callus. The selectable marker gene may provide antibiotic or herbicide resistance to the rice cells, or allow the utilization of substrates, for example mannose, for growth. The selectable marker preferably confers geneticin, hygromycin or phosphinothricin resistance to the rice cells. The regenerable rice cells are preferably from the scutellum of immature embryos, mature

10   embryos, callus derived from these, or the meristematic tissue. Transformed cells are selected and then regenerated by methods well known in the art, such as described in Example 2, to produce transformed rice plants.

The transformed plant may contain a selectable marker gene, or such gene may be

15   removed during or after regeneration, for example by excision of the selectable marker gene out of the genome or by segregation of the selectable marker gene away from the transgene that leads to inhibition of *SBEIIa* and/or *SBEIIb*.

Plants where the transgene or mutation has been integrated into a chromosome can be

20   screened for by, for example, using a suitable nucleic acid probe specific for the transgene or phenotypic observation. Any of several methods may be employed to determine the presence of a transformed plant. For example, polymerase chain reaction (PCR) may be used to amplify sequences that are unique to the transformed plant, with detection of the amplified products by gel electrophoresis or other methods. DNA may be extracted from

25   the plants using conventional methods and the PCR reaction carried out using primers that will distinguish the transformed and non-transformed plants. For example, primers may be designed that will amplify a region of DNA from the transformation vector reading into the construct and the reverse primer designed from the gene of interest. These primers will only amplify a fragment if the plant has been successfully transformed. An alternative

30   method to confirm a positive transformant is by Southern blot hybridization, well known in the art. Plants which are transformed or mutant may also be identified i.e. distinguished from non-transformed or wild-type plants by their phenotype, for example conferred by the presence of a selectable marker gene, or the presence of a particular protein by immunological methods, or by the absence of a protein, for example that absence of the

35   SBEIIa protein in the endosperm as detected by ELISA assay or Western blot analysis. An indication used in screening such plants might also be by observation of the

phenotypic traits of the grain, for example by visual inspection or measurement of
shrunken grain, or testing for elevated amylose content, or checking microscopically for
the presence of birefringence of starch granules.

5   *Mutation*

Introduction of the genetic variation leading to reduced activity of the SBEIIa and SBEIIb
enzymes or other starch biosynthetic enzyme in the rice endosperm may also be achieved
by the appropriate mutations within the respective gene or regulatory sequences of the
gene. The extent to which the gene is inhibited will to some degree determine the

10   characteristics of the starch made. The mutations may be truncation or null mutants and
these are known to have a significant impact on the nature of the starch, however an altered
starch structure will also result from a leaky mutant that sufficiently reduces amylopectin
synthesis enzyme activity to provide the characteristic of interest in the starch or grain of
rice. Other chromosomal rearrangements may also be effective and these might include

15   deletions, inversions, duplication or point mutations.

Mutagenesis can be achieved by chemical or radiation means, for example EMS or sodium
azide (Zwar and Chandler, 1995) treatment of seed, or gamma irradiation. For gamma ray
induced mutation, seeds may be irradiated at a dose of 20-50 kR from a ^{60}Co source

20   (Zikiryaeva and Kasimov, 1972). EMS mutagenesis may be performed by treating the
seeds with EMS (0.03%, v/v) as per Mullins et al., (1999). Isolation of mutants may be
achieved by screening mutagenised plants or seed. For example, a mutagenized population
of rice may be screened for high amylose content in the grain and/or longer than normal
amylopectin chain length distribution, or loss of the SBEIIa and/or SBEIIb proteins by

25   ELISA, or for altered grain morphology (Green et al., 1997). Screening is preferably
done in a rice genotype that already lacks one of the SBE activities, for example in a
SBEIIa- or SBEIIb-negative background. Such mutations may then be introduced into
desirable genetic backgrounds by crossing the mutant with a plant of the desired genetic
background and performing a suitable number of backcrosses to cross out the originally

30   undesired parent background. Preferred mutations are those which affect the expression
or activity of both SBEIIa and SBEIIb in rice.

The invention thereby provides high amylose, non-transgenic rice grain and products
therefrom.

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Mutations in the genes encoding the SBEIIa, SBEIIb or other enzymes involved in
amylopectin synthesis, for example increased levels of GBSS, provide an increased
proportion of amylose in starch of the rice endosperm. The amount of amylose per
individual grain may be increased as a consequence of diverted carbon flow from
5   amylopectin to amylose, or it may be decreased if there is a significant decrease in starch
production per grain. In either case, the relative level of amylose as a percentage of starch
increases.

Seed with starch granules having a distorted shape have been reported in high amylose
10   barley (Morell et al, 2003) and in low amylopectin (LAPS) maize having about 90%
amylose in starch (Sidebottom et al., 1998). This phenotype can be used in screening a
mutagenised population of rice. Birefringence can also be used for this. Birefringence is
the ability of a substance to refract light in two directions; this produces a dark cross called
a "maltese cross" on each starch granule when viewed with a polarizing microscope.
15   Birefringence is an indicator of the degree of ordered structural organization of the
polymers within the granules (Thomas and Atwell, 1999). Loss of birefringence in starch
granules is generally well correlated with increased amylose content.

Suitable for food production
20   In another aspect, the invention provides rice that is useful for food production, the grain
having starch comprising a high relative amylose content and a reduced amylopectin
content. Preferably the rice plant from which the grain is obtained has a reduced level of
SBEIIa and SBEIIb proteins and/or activities in the endosperm during development. The
rice plant of the present invention is useful for food production and in particular for
25   commercial food production.

The desired genetic background of the rice will include considerations of agronomic yield
and other characteristics. Such characteristics might include agronomic performance,
disease resistance and abiotic stress resistance. In Australia one might want to cross the
30   altered starch trait into rice cultivars such as Amaroo, Ali Combo, Basmati, Bogan, Bombia,
Doongara, Goolarah, Illabong, Jarrah, Koshihikari, Kyeema, Langi, Millin, Namage, Opus,
Pelde or other commonly grown varieties. The examples provided are suitable for an
Australian production region, and other varieties will be suited for other growing regions.
It is preferred that the rice variety of the invention provide a yield not less than 80% of the
35   corresponding wild-type variety in at least some growing regions, more preferably not less

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than 90% and even more preferably not less than 95%. The yield can readily be measured in controlled field trials.

5   The starch content of the grain should be at least about 25%, preferably at least 35% or 45% and more preferably near to the wild-type levels of 55 to 65% (w/w). Most preferably, the grain has a starch content of at least 90% that of grain from an equivalent, but unaltered, rice. Lower starch contents than wild-type are likely a consequence of reduced amylopectin levels. Even with lower starch contents, the grain may still be useful for commercial food production because of the relatively high value of the high amylose
10   products. Other desirable characteristics include the capacity to mill the grain, in particular the grain hardness. Another aspect that might make a rice plant of higher value is the degree of starch extraction from the grain, the higher extraction rates being more useful. Grain shape is also another feature that can impact on the commercial usefulness of a plant, thus grain shape can have an impact on the ease or otherwise with which the grain
15   can be milled. For example, an elongated grain morphology may make it difficult to mill and process.

Starch may be readily isolated from rice grain using standard methods, for example by wet milling of brokers with alkali solution (sodium hydroxide) to remove protein. Brokens are
20   steeped in alkali solution for 24 hours and are then wet milled in pin mills, hammermills or stone-mill disintegrators with the alkali solution. After the batter is stored for 10 to 24 hours, fiber is removed by passing it through screens, the starch collected by centrifugation, washed thoroughly with water and dried.

25   *Physical characteristics of the altered starch*
     In another aspect of the invention, the rice starch may have an altered gelatinisation temperature, which may be readily measured by differential scanning calorimetry (DSC). Gelatinisation is the heat-driven collapse (disruption) of molecular order within the starch granule in excess water, with concomitant and irreversible changes in properties such as
30   granular swelling, crystallite melting, loss of birefringence, viscosity development and starch solubilisation. The gelatinisation temperature may be either increased or decreased compared to starch from wild-type plants, depending on the chain length of the remaining amylopectin. High amylose starch from *ae* (amylose extender) mutants of maize showed a higher gelatinisation temperature than normal maize (Fuwa et al., 1999, Krueger et al.,
35   1987). On the other hand, starch from barley *sex6* mutants that lack starch synthase IIa

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activity had lower gelatinisation temperatures and the enthalpy for the gelatinisation peak was reduced when compared to that from control plants (Morell et al., 2003).

The altered gelatinisation temperature may be in addition to the relatively high amylose

5   content. The gelatinisation temperature of wild-type rice starch is typically about 61-67°C (Rahman et al, 2000) for the temperature of the first peak, defined as the onset temperature, as measured by differential scanning calorimetry.

The starch may also be characterized by its swelling rate in heated excess water compared

10   to wild-type starch. Swelling volume is typically measured by mixing either a starch or flour with excess water and heating to elevated temperatures, typically greater than 90°C. The sample is then collected by centrifugation and the swelling volume is expressed as the mass of the sedimented material divided by the dry weight of the sample. A low swelling characteristic is useful where it is desired to increase the starch content of a food

15   preparation, in particular a hydrated food preparation.

The starch structure of the rice of selected forms of the present invention may also differ in that the degree of crystallinity is reduced compared to normal starch isolated from rice. The reduced crystallinity of a starch is also thought to be associated with enhance

20   organoleptic properties and contributes to a smoother mouth feel. Thus the starch may additionally exhibit reduced crystallinity resulting from reduced levels of activity of one or more amylopectin synthesis enzymes. Crystallinity is typically investigated by X-ray crystallography.

25   One measure of an altered amylopectin structure is the distribution of chain lengths, or the degree of polymerization, of the starch. The chain length distribution may be determined by using fluorophore-assisted carbohydrate electrophoresis (FACE) following isoamylase de-branching. The amylopectin of the starch of the invention may have a distribution of chain length in the range from 5 to 60 that is greater than the distribution of starch from

30   wild-type plants upon debranching. Starch with longer chain lengths will also have a commensurate decrease in frequency of branching. Thus the starch may also have a distribution of longer amylopectin chain lengths in the amylopectin still present.

Food characteristics

35   Rice starch is a major source of carbohydrate in the human diet, particularly in Asia, and the grain of the invention and products derived from it can be used to prepare food. The

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food may be consumed by man or animals, for example in livestock production or in pet-food. The grain derived from the altered rice plant can readily be used in food processing procedures, and therefore the invention includes milled, ground, kibbled, cracked, rolled, boiled or parboiled grain, or products obtained from the processed or whole grain of the

5   rice plant, including flour, brokers, rice bran and oil. The products may be precooked or quick-cooking rice, instant rice, granulated rice, gelatinized rice, canned rice or rice pudding. The grain or starch may be used in the production of processed rice products including noodles, rice cakes, rice paper or egg roll wrapper, or in fermented products such as fermented noodle or beverages such as sake. The grain or starch derived therefrom may

10   also be used in, for example, breads, cakes, crackers, biscuits and the like, including where the rice flour is mixed with wheat or other flours, or food additives such as thickeners or binding agents, or to make drinks, noodles, pasta or quick soups. The rice products are suitable for use in wheatfree diets. The grain or products derived from the grain of the invention are particularly desired in breakfast cereals such as puffed rice, rice flakes or as

15   extruded products. The high amylose starches of the invention can also be used to form high strength gels that are useful in the confectionery industry, or allow lower molding and curing times. They may also be used as a coating, for example to reduce oil absorption in deep-fried potato or other foods.

20   *Dietary fiber*
        Dietary fibre, in this specification, is the carbohydrate and carbohydrate digestion products that are not absorbed in the small intestine of healthy humans but enter the large bowel. This includes resistant starch and other soluble and insoluble carbohydrate polymers. It is intended to comprise that portion of carbohydrates that are fermentable, at least partially, in

25   the large bowel by the resident microflora.

The starch of the invention preferably contains relatively high levels of dietary fiber, more particularly amylose. The dietary fiber content of the grain of the present invention may or may not result solely from the increased relative endospermal amylose content.

30
        Aspects of this invention might also arise from the combination of aleurone layer and germ in combination with high levels of dietary fiber. Specifically, this may arise where higher relative levels of aleurone or germ are present in the grain. Where the rice grain is slightly shrunken the endosperm is present in reduced amounts and the aleurone layer and

35   the germ are present in relatively elevated amounts. Thus the rice has a relatively high level of certain beneficial elements or vitamins in combination with elevated resistant starch,

such elements include divalent cations, bioavailable Ca^{++} and vitamins such as folate or antioxidants such as tocopherols or tocotrienols. One specific form of milled product might be one where the aleurone layer is included in the milled product. Particular milling process might be undertaken to enhance the amount of aleurone layer in the milled

5   product. Thus any product derived from grain milled or otherwise processed to include aleurone layer and germ will have the additional nutritional benefits, without the requirement of adding these elements from separate sources.

Resistant starch

10   Resistant starch is defined as the sum of starch and products of starch digestion not absorbed in the small intestine of healthy humans but entering into the large bowel. Thus, resistant starch excludes products digested and absorbed in the small intestine. Resistant starches include physically inaccessible starch (RS1 form), resistant native starch granules (RS2), retrograded starches (RS3), and chemically modified starches (RS4). The altered

15   starch structure and in particular the high amylose levels of the starch of the invention give rise to an increase in resistant starch when consumed in food. The starch may be in an RS1 form, being somewhat inaccessible to digestion. Starch-lipid association as measured by V-complex crystallinity is also likely to contribute to the level of resistant starch.

20   It will be understood that one benefit of the present invention is that it provides for products that are of particular nutritional benefit, and moreover it does so without the need to post-harvest modify the starch or other constituents of the rice grain. However it may be desired to make modifications to the starch or other constituent of the grain, and the invention encompasses such a modified constituent. Methods of modification are well

25   known and include the extraction of the starch or other constituent by conventional methods and modification of the starches to increase the resistant form. The starch may be modified by treatment with heat and/or moisture, physically (for example ball milling), enzymatically (using for example α– or β-amylase, pullalanase or the like), chemical hydrolysis (wet or dry using liquid or gaseous reagents), oxidation, cross bonding with

30   difunctional reagents (for example sodium trimetaphosphate, phosphorous oxychloride), or carboxymethylation.

Glycemic index

Glycaemic Index (GI) relates to the rate of digestion of foods comprising the starch, and is

35   a comparison of the effect of a test food with the effect of white bread or glucose on excursions in blood glucose concentration. The Glycaemic Index is a measure of the likely

effect of the food concerned on post prandial serum glucose concentration and demand for insulin for blood glucose homeostasis. One important characteristic provided by foods of the invention is a reduced glycaemic index. Serum glucose levels were lower 30 min after ingestion of high amylose rice products by human volunteers compared to low amylose

5   rice (Goddard et al., 1984). Furthermore, the foods may have a low level of final digestion and consequently be relatively low-calorie. A low calorific product might be based on inclusion of flour produced from milled rice grain. Such foods may have the effect of being filling, enhancing bowel health, reducing the post-prandial serum glucose and lipid concentration as well as providing for a low calorific food product.

10

Non-food applications
The present invention provides modified or improved starches having elevated levels of amylose or reduced levels of amylopectin whose properties satisfy any of various industrial requirements. Starch is widely used in non-food industries, including the film,

15   paper, textile, corrugating and adhesive industries (Young, 1984), for example as a sizing agent. Rice starch may be used as a substrate for the production of glucose syrups or for ethanol production. The physical properties of unmodified starch limits its usefulness in some applications and often imposes a requirement for chemical modification that can be expensive or have other disadvantages. The invention provides starch for which less post-

20   harvest modification may be required, in particular due to the reduced amylopectin content in combination with other physical properties. For example, the pasting temperature, resistance to shearing stresses, film strength and/or water resistance of starches and product made from the grain of this invention may be altered. The starch may also be used to prepare a biodegradable loose-fill packing material that can be used as a

25   replacement for polystyrene or other packing material.

It will be understood that whilst various indications have been given as to aspects of the present invention, the invention may reside in combinations of two or more aspects of the present invention.

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EXAMPLES

<u>Example 1. Materials and Methods</u>

Materials and media

5   <u>N6 macro-elements (20X stock solution)</u>

	g/l
$(NH_4)_2SO_4$	9.3
KNO_3	56.6
KH_2PO_4	8
$MgSO_4.7H_2O$	3.7
$CaCl_2.2H_2O$	3.3

<u>MS macro-elements (20X stock solution)</u>

	g/l
NH_4NO_3	33.0
KNO_3	38.0
KH_2PO_4	3.4
$MgSO_4.7H_2O$	7.4
$CaCl_2.2H_2O$	8.8

<u>N6 micro-elements (1000X stock solution)</u>

	mg/100 ml
$MnSO_4.4H_2O$	440
$ZnSO_4.7H_2O$	150
H_3BO_3	160
KI	80

<u>MS micro-elements (1000X stock solution)</u>

	mg/l
$MnSO_4.4H_2O$	22300
$Na_2MoO_4.2H_2O$	250
H_3BO_3	6220
$ZnSO_4.7H_2O$	8600
$CuSO_4.5H_2O$	25
$CoCl_2.6H_2O$	25
KI	830

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B5 micro-elements (100X stock solution)

		mg/l
	$MnSO_4.4H_2O$	1000
5	$Na_2MoO_4.2H_2O$	25
	H_3BO_3	300
	$ZnSO_4.7H_2O$	200
	$CuSO_4.5H_2O$	3.87
	$CoCl_2.6H_2O$	2.5
10	KI	75

N6 vitamins (100X stock solution)

		mg/100 ml
	Glycine	20
15	Thiamine-HCl	10
	Pyridoxine-HCl	5
	Nicotinic acid	5

MS vitamins (100X stock solution)

20		mg/100 ml
	myo-Inositol	1000
	Thiamine-HCl	1
	Pyridoxine-HCl	5
	Nicotinic acid	5

25

B5 vitamins (100X stock solution)

		mg/100 ml
	Glycine	1000
	Thiamine-HCl	100
30	Pyridoxine-HCl	10
	Nicotinic acid	10

MS iron (200X stock solution)
ml/500 ml

	$FeCl_3$ (60% w/v)	2.7

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MS Na_2.EDTA (200X stock solution)

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g/500 ml

Na$_2$.EDTA   3.7

A 2,4- dichloro-phenoxyacetic acid (2,4-D) (1mg/ml, Sigma No. D-6679) stock solution
was prepared by dissolving 100mg of 2,4-D in1ml absolute ethanol, adding 3ml of 1N
KOH, and adjusting the pH to 6 with 1N HCl.
Solutions of 6-benzyl amino purine (1mg/ml BAP, Sigma No. B-3408) and naphthalene
acetic acid (1mg/ml NAA, Sigma No.N-0640) were prepared.
Abscisic acid (ABA, 2.5mg/ml, Sigma No. A-1049) was prepared by dissolving 250mg
ABA in 2 ml of 1M NaOH, making up to 100 ml with sterile water.
Timentin (150mg/ml, Smith-Kline Beecham 6571-30) was prepared by dissolving 3.1g in
20.66ml of sterile water.
Hygromycin (50 mg/ml) was obtained from Roche (No. 843 555) and other reagents from
Sigma.

N6D media for callus induction

	amount/litre
N6 macro (20X)	50 ml
N6 micro (1000X)	1 ml
N6 vitamins (100X)	10 ml
MS iron (200X)	5 ml
MS Na$_2$ EDTA (200X)	5 ml
Myoinositol	100 mg
Casamino acid	300 mg
Proline	2.9 g
2,4-D (1mg/ml)	2 ml
Sucrose	30 g

The pH was adjusted to 5.8 with 1M KOH, 3 g phytogel added per liter and the mixture
autoclaved.

NB media for subculturing

	amount/liter
N6 macro-elements (20X)	50 ml
B5 micro-elements (100X)	10 ml
B5 vitamins (100X)	10 ml

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MS iron (200X)	5 ml
MS Na$_2$ EDTA (200X)	5 ml
2,4-D (1mg/ml)	2 ml
Sucrose	30 g
Proline	500 mg
Glutamine	500 mg
Casein enzymatic hydrolysate (CEH)	300 mg

The pH was adjusted to 5.8-5.85 with 1M KOH, 3 g phytogel added per liter, and the mixture autoclaved.

MS media for subculturing

	amount/liter
MS macro-elements (20X)	25 ml
MS micro-elements (1000X)	1 ml
MS vitamins (100X)	10 ml
MS iron (200X)	5 ml
MS Na$_2$ EDTA (200X)	5 ml
Sucrose	10 g

The pH was adjusted to 5.8-5.85 with 1M KOH, 2.5 g phytogel added per liter, and the mixture autoclaved.

NBO: NB media, plus 30 g/l mannitol and 30 g/l sorbitol, added before pH adjustment.

NBHT30: NB media plus 30 mg/l hygromycin and 150 mg/l Timentin added after autoclaving and just before pouring.

NBHT50: NB media plus 50 mg/l hygromycin and 150 mg/l Timentin added after autoclaving and just before pouring.

PRHT50: NB media (with no 2,4-D), plus following added after autoclaving, to a final concentration of: BAP (2 mg/l), NAA (1 mg/l), ABA (5 mg/l), Hygromycin (50 mg/l) and Timentin (150 mg/l).

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RHT50: NB media (with no 2,4-D), plus following added after autoclaving, to a final concentration of: BAP (3 mg/l), NAA (0.5 mg/l), Hygromycin (50 mg/l), Timentin (150 mg/l).

5   MST medium: MS medium, with 0.05 mg/l NAA and 150 mg/l Timentin added after autoclaving.

Rice transformation

Mature grains were dehusked, soaked in 70% ethanol for 1 minute and washed with sterile
10   water 3 times before being soaked in 50% bleach for 30 min. The sterilized grain were washed thoroughly with sterile water under aseptic conditions and then plated onto N6D medium. Plates were sealed with Micropore-tape and incubated under light for 6-8 weeks at 26-28°C for callus production. Callus was produced, presumably from the scutellum of the embryos, without any dissection of the embryos from the grains. If subculture was
15   required, calli were transferred on to NB medium and the plates sealed with parafilm. The plates were left in the dark at 28°C in a box covered with aluminum foil. Subcultures were carried out every 4 weeks. Calli were subcultured not more than 5 times before use for transformation.

20   For *Agrobacterium* mediated transformation, *Agrobacterium* strains containing the gene constructs to be transferred were grown at 28 °C on plates with appropriate antibiotics, and after 2 days growth, the cells scraped off the plates and resuspended in liquid NB medium containing 100 μM acetosyringone. Healthy looking calli were immersed in the bacterial suspension for 10 min, the calli then drained briefly and placed on NBO plates containing
25   100 μM acetosyringone in the dark at 25 °C for 2 days. This period is referred to as "co-cultivation" in the presence of *Agrobacterium* containing the gene construct. After co-cultivation, the calli were washed in sterile water containing 150 mg/l Timentin, blotted dry briefly, and plated onto NBHT30 (which includes the selective agent hygromycin) plates containing 150 mg/l Timentin. After 3-4 weeks at 26-28 °C, any calli showing zones of
30   growth were subcultured onto the same medium for a further 10-24 days. Sustained growth indicated calli resistant to hygromycin, i.e. transformed calli. These calli were transferred onto NBHT50 plates containing timentin and incubated at 26-28 °C in the dark for a further 14-21 days. Healthy looking calli were transferred to PRHT50 plates for a further 8-12 days in the dark. Finally, shoots were regenerated on RHT50 medium in the
35   light at 28 °C for 30 days, or more. Shoots that showed root formation were transferred to 1/2MST medium and when large enough transferred to soil in the greenhouse. This

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method has proven successful with a variety of rice cultivars including both japonica and indica types.

Example 2. Construct preparation for gene down-regulation.

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Segments of the rice *SBEI*, *SBEIIa* and *SBEIIb* genes were amplified by PCR for use in preparation of gene constructs for the down-regulation of gene expression in rice. The segments chosen were from exon regions near the 3'ends of the genes as these regions of the genes are more divergent and this was thought to reduce the likelihood of cross-

10   silencing of the genes by the constructs in transformed rice. The segments amplified were: SBEI- nucleotides 1982-2527 of GenBank accession No. D11082; SBEIIa- nucleotides 2458-2997 of Accession No. AB023498; SBEIIb- nucleotides 2414 to 2912 of Accession No. D16201 (Sequences shown in Figures 1-3). The amplified fragments, which contained additional sequences comprising restriction endonuclease sites at the ends for

15   convenience in subsequent cloning steps, were cloned into the plasmid vector pGEM®-T. An intron sequence was also obtained by amplifying the SBEI intron 9 sequence from rice. The fragment included the sequence from nucleotides 9112-9606 of the genomic sequence Genbank Accession No. D10838 and flanking *Spe*I and *Eco*RI restriction sites, and was inserted into pBCSK⁻ (Stratagene) to form pRint9_BC (Figure 4). The exon

20   fragments from the SBEI, SBEIIa and SBEIIb genes were then cloned in the antisense and sense orientations in pRint9_BC using the *Spe*I/*Xba*I and *Xho*I/*Eco*RI sites, respectively. This served to form an inverted repeat for each of these sequences, each separated by the intron sequence. The resultant plasmids were designated pRBEI.IR, pRBEIIa.IR, and pRBEIIb.IR (Figure 5). The chimeric fragments were excised with *Bam*HI and *Kpn*I and

25   inserted into the same sites of pBx17casNOT (Figure 6). This joined the antisense/intron/sense chimeric fragments to the Bx17 promoter region and the nos3' termination region in the correct orientation for expression. Each expression cassette was then excised by digestion with *Hind*III and *Not*I and inserted into the binary vector pWBvec8 (Wang et al. Acta Hort 461:401-407, 1998) which contains a plant expressible

30   hygromycin gene for selection in plant cells as well as a spectinomycin resistance gene for selection in bacteria. The constructs were designated dsSBEI, dsSBEIIa, and dsSBEIIb. These constructs were then transferred to the *Agrobacterium tumefaciens* strain (AGL1) cells (Lazo et al. (1991)) by electroporation.

35   A further duplex-RNA (dsRNA) construct was made to reduce the expression of the *SBEIIa* and possibly the *SBEIIb* genes of rice, using sequences from the corresponding

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SBEIIa gene from wheat. As for the other constructs, above, the desired nucleic acid
sequence corresponding to part of the *SBEIIa* gene occurred in both the sense and
antisense orientations relative to the promoter so that the expressed RNA comprised
complementary regions that were able to basepair and form a duplex or double-stranded

5   RNA. A spacer region between the sense and antisense sequences comprised an intron
sequence which, when transcribed as part of the RNA in the transformed plant, would be
spliced out to form a tight "hairpin" duplex structure. The inclusion of an intron has
been found to increase the efficiency of gene silencing conferred by duplex-RNA
constructs (Smith et al, 2000). The desired nucleic acid was linked to a high molecular

10   weight glutenin (HMWG) promoter sequence from wheat and terminator sequence from
the nopaline synthase gene from *Agrobacterium* (nos3'). This provided endosperm
specific expression of the dsRNA sequence.

The SBEIIa duplex-RNA construct contained 1536bp of nucleotide sequence amplified by

15   PCR from the wheat *SBEIIa* gene (GenBank Accession number AF338431). This
included: a 468bp sequence that comprised the whole of exons 1 and 2 and part of exon 3,
with *Eco*RI and *Kpn*I restriction sites on either side (fragment 1), a 512bp sequence
consisting of part of exons 3 and 4 and the whole of intron 3 of *SBEIIa* with *Kpn*I and
*Sac*I sites on either side (fragment 2) and a 528bp fragment consisting of the complete

20   exons 1, 2 and 3 of *SBEIIa* with *Bam*HI and *Sac*I sites on either side (fragment 3). The
sequences used have 80% identity over 217 nucleotides with the rice *SBEIIa* gene (SBE4),
including greater homology over shorter regions (87% over 50 nucleotides and 92% over
27 nucleotides), and it was therefore expected that expression of this sequence in rice
endosperm would lead to significant decrease in the expression of rice *SBEIIa*. The wheat

25   sequence was also 76% identical over 113 nucleotides to rice-branching enzyme-3, the
equivalent of *SBEIIb*, and this was expected to affect the level of this transcript as well.

Fragments 1, 2 and 3 were then ligated so that the sequence of fragment 3 was ligated to
fragment 2 in the antisense orientation relative to fragment 1. The duplex-RNA constructs

30   were initially generated in the vector pBx17casNOT (Figure 6) which contained the
HMWG promoter sequence and nos3' terminator. The gene construct in this vector was
designated pBx17ds-wSBEIIa and the duplex-RNA gene designated ds-wSBEIIa. The
cassette including the ds-wSBEIIa gene was inserted into pWBvec8, introduced into
Agrobacterium strain AGL1 and used to transform rice as described in Example 1.

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Example 3. Production of rice with reduced SBE activity.

The constructs dsSBEI, dsSBEIIa, dsSBEIIb and ds-wSBEIIa in AGL1 cells were used to
produce transformed rice plants (cv. Nipponbare) according to the methods described in
5   Example 1. Five hundred rice calli were used for each construct, transformed calli selected
and rice plants regenerated. After the plants were transferred to soil, transformation of the
plants was demonstrated by PCR or Southern blot hybridization analysis using primers or
probes specific for the SBEI, SBEIIa or SBEIIb gene segments used. Of 24 regenerated
plants from the transformation with ds-wSBEIIa, 21 were shown to be positive for the
10   introduced SBEIIa sequences.

Grain from the transformed plants (T1 seed) is assayed for SBE proteins by Western blot
analysis using specific antibodies to the respective proteins, after gel electrophoresis of
endosperm proteins on acrylamide gels. SBE activity is reduced in the majority of
15   transformed lines. The proportion of amylose in starch of the grain is determined. Some
of the SBEIIa transformed lines show relative amylose levels of at least 40%, and some of
these more than 50%. The proportion of amylose is raised even further when both SBEIIa
and SBEIIb activities are reduced.

20   **Example 4. Starch and protein analysis**

Carbohydrate determination and analysis
Starch is isolated from developing endosperm or from mature grain using the methods of
Takeda et al., (1986); Lumdubwong et al., (2000); Chiou et al (2002) orSchulman *et al.*,
25   (1991). Starch content is determined using the total starch analysis kit supplied by
Megazyme (Bray, Co Wicklow, Republic of Ireland). The starch content is then compared
to control plants. Subtraction of the starch weight from the total grain weight to give a
total non-starch content of the grain determines whether the reduction in total weight is due
to a reduction in starch content.
30
The amylose content of starch samples is determined by the colorimetric (iodometric)
method of Morrison and Laignelet (1983) with slight modifications as follows.
Approximately 2 mg of starch is weighed accurately (accurate to 0.1 mg) into a 2 ml
screw-capped tube fitted with a rubber washer in the lid. To remove lipid, 1 ml of 85%
35   (v/v) methanol is mixed with the starch and the tube heated in a 65°C water bath for 1 hour
with occasional vortexing. After centrifugation at 13,000g for 5 min, the supernatant is

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carefully removed and the extraction steps repeated. The starch is then dried at 65°C for 1 hour and dissolved in urea-dimethyl sulphoxide solution (UDMSO; 9 volumes of dimethyl sulphoxide to 1 volume of 6 M urea), using 1 ml of UDMSO per 2 mg of starch (weighed as above). The mixture is immediately vortexed vigorously and incubated in a

5   95°C water bath for 1 hour with intermittent vortexing for complete dissolution of the starch. An aliquot of the starch-UDMSO solution (50 µl) is treated with 20 µl of I_2-KI reagent that contains 2 mg iodine and 20 mg potassium iodide per ml of water. The mixture is made up to 1 ml with water. The absorbance of the mixture at 650 nm is measured by transferring 200 µl to a microplate and reading the absorbance using an

10   Emax Precision Microplate Reader (Molecular Devices, USA). Standard samples containing from 0 to 100% amylose and 100% to 0% amylopectin are made from potato amylose and corn (or potato) amylopectin (Sigma) and treated as for the test samples. The amylose content (percentage amylose) is determined from the absorbance values using a regression equation derived from the absorbances for the standard samples. Analysis of

15   the amylose/amylopectin ratio of non-debranched starches may also be carried out according to Case et al., (1998) or by an HPLC method for separating debranched starches as described by Batey and Curtin (1996).

The distribution of chain lengths in the starch may be analysed by fluorophore assisted

20   carbohydrate electrophoresis (FACE) using a capillary electrophoresis unit according to Morell et al (1998), after debranching of the starch samples. The gelatinisation temperature profiles of starch samples may be measured in a Pyris 1 differential scanning calorimeter (Perkin Elmer, Norwalk CT, USA). The viscosity of starch solutions may be measured on a Rapid-Visco-Analyser (RVA, Newport Scientific Pty Ltd, Warriewood,

25   Sydney), for example using conditions as reported by Batey et al., 1997. The parameters that may be measured include peak viscosity (the maximum hot paste viscosity), holding strength, final viscosity and pasting temperature. The swelling volume of flour or starch may be determined according to the method of Konik-Rose et al (2001). The uptake of water is measured by weighing the sample prior to and after mixing the flour or starch

30   sample in water at defined temperatures and following collection of the gelatinized material.

β-Glucan levels may be determined using the kit supplied by Megazyme (Bray, Co Wicklow, Republic of Ireland).

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Analysis of protein expression in endosperm.
Specific protein expression in endosperm is analyzed by Western blot procedures.
Endosperm is dissected away from all maternal tissues and samples of approximately 0.2

5   mg are homogenized in 600μl of 50 mM KPi buffer (42 mM K_2HPO_4 and 8 mM
KH_2PO_4), pH 7.5, containing 5 mM EDTA, 20% glycerol, 5 mM DTT and 1 mM
Pefabloc. The ground samples are centrifuged for 10 min at 13,000g and the supernatant
aliquoted and frozen at –80°C until use. For total protein estimation, a BSA standard curve
is set up using 0, 20, 40, 60, 80 and 100 μl aliquots of 0.25 mg/ml BSA standard. The

10   samples (3μl) are made up to 100μl with distilled water and 1 ml of Coomassie Plus
Protein reagent is added to each. The absorbance is read after 5 min at 595nm, using the
zero BSA sample from the standard curve as the blank, and the protein levels in the
samples determined. Samples containing 20 μg total protein from each endosperm are run
on an 8% non denaturing polyacrylamide gel containing 0.34 M Tris-HCl (pH 8.8),

15   acrylamide (8.0%), ammonium persulphate (0.06%) and TEMED (0.1%). Following
electrophoresis, the proteins are transferred to a nitrocellulose membrane according to
Morell et al., (1997) and immunoreacted with SBEIIa or SBEIIb specific antibodies.

Example 5. Optimised gene silencing of target genes by identification of unique
20   **sequences in the rice genome.**

Genetic sequences used to reduce target gene expression (gene silencing) by methods
such as, for example, using duplex RNA, antisense or co-suppression constructs, are
preferably highly specific for the target gene. That is, the silencing molecule comprises a

25   nucleotide sequence of at least 19 consecutive nucleotides that is at least about 95%
identical to a sequence of at least 19 consecutive nucleotides of the target gene or its
complement. Ideally, for maximum specificity, the targeted sequence is unique to the target
gene and is not present elsewhere as an expressed gene in the plant genome. This would
minimize "off-gene effects". We have used knowledge of the near complete rice genome

30   sequence to compare the SBE target gene sequences with the remainder of the rice genome
to identify optimized target sequences within these genes.

A rice genomic DNA sequence database (OSA1.seq) was downloaded from the TIGR
website (http://www.tigr.org/tdb/e2k1/osa1/) in FASTA format. The database was
35   formatted and made available for BLAST using "formatdb" and for EMBOSS function
seqret using "dbifasta". Query sequences were created in a FATSA format and used to

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search for homologous sequences in the rice genome by running a BLAST based Gene Silencing program (P. Waterhouse et al, CSIRO Plant Industry, personal communication) with a set of preset parameters (Options for compare: word 19 and stringency 18).

5   The SBEIIa, SBEIIb and SBEI sequences used to prepare gene silencing constructs as described in Example 2 were used as query sequences against the rice genome. The output is shown in Figure 8. Multiple "NNNN..." in the output sequences indicated that the query sequences in those regions had homology in a region of at least 19 consecutive nucleotides with a sequence elsewhere in the rice genome. It can be seen that the SBEIIa

10   sequence used is unique, the SBEIIb sequence used contains some non-unique sequence, while the SBEI sequence used appears to be duplicated elsewhere in the rice genome except for the terminal 57 nucleotides which appears to be clear (Figure 8). Examination of the current rice genome sequence revealed that there appears to be an overlapping genomic DNA sequence between two overlapping BAC clones which may include the

15   *SBEI* gene region. The apparent duplication may be real or may represent an error in the assembly of the rice genome in that region.

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